IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of Reinhard Zeissig et al.

Serial No. 09/873,952

Filed on June 4, 2001

For MEANS OF TUMOR THERAPY

Attorney's Docket -107-032

Commissioner of Patents
Washington DC 20231

Sir:

PRELIMINARY AMENDMENT

Prior to taking up this application for action, please enter the following amendment.

In the disclosure

Please replace the originally filed disclosure and abstract, with the enclosed substitute disclosure and abstract.

In the claims

Delete claims 1-8.

Add the following new claims:

-- 9. A composition containing an antineoplastic alkylphospholipid, and an antineoplastic antiestrogen in a lipid vesicle.--

-- 10. A liposome composition which comprises

(i) an antineoplastic alkylphospholipid,

(ii) an antineoplastic water -or lipid-soluble antiestrogen associated in liposomal form with said antineoplastic alkylphospholipid,

(iii) a nonneoplastic phospholipid, and

optionally one or more of (iv) a sterol, (v) a positively or negatively charged lipid, and (vi) a PEG lipid.--

-- 11. The composition of claim 10, wherein said alkylphospholipid has the formula

R-Y-P-X

wherein

R is a C_{12-22} alkyl, alkenyl, or alkinyl residue,

Y is oxygen, sulfur, or a CH_2 residue,

P is a PO_2 residue, and

X is a choline, or modified choline residue, or serine, ethanolamine, or glycerine group, or a synthetic modification thereof . –

-- 12. The composition of claim 10, wherein said alkylphospholipid is hexadecylphosphoicholine, octadecylphosphocholine, erucylphosphocholine, octadecyl-[2-(N-methylpiperidino)ethyl]phosphate,

octadecylphosphoethanolamine, or hexadecyl-phosphoserine.--

-- 13. The composition of claim 10, wherein said antiestrogen is tamoxifen, droloxifen, toremifen, idoxiphen, raloxiphen, miproxiphen-phosphate (TAT-59), ICI 164,3384, ICI 182,780, a main metabolite of tamoxiphen, 4-hydroxy tamoxiphen, N-desmethyltamoxiphen.--

-- 14. The composition of claim 10, wherein said nonneoplastic phospholipid is a naturally occurring or synthetic material, with a lipid : antiestrogen molar ratio of between 0-10 : 1 (m/m). --

-- 15. The composition of claim 14, wherein said nonneoplastic phospholipid is phosphocholine, serine, ethanolamine, glycerol.--

-- 16. The composition of claim 10, wherein said sterol is cholesterol, or sitosterol in a sterol : alkylphospholipid molar ratio of 0-1 : 1.--

-- 17. The composition of claim 10, wherein said PEG lipid is N-(O-methoxy-polyethyleneglycyl)-1,2-distearyl-s,n-glycero-3-phosphoethanolamine (PEG_{2000} -

DSPE).--

-- 18. The composition of claim 10, wherein said antineoplastic phospholipid is OPP, and said antineoplastic antiestrogen is tamoxiphen. --

REMARKS

Claims 9-18 are in the application.

Also enclosed herewith is a comparison copy of the substitute disclosure showing the changes. No new matter was added.

Favorable consideration of the application, as amended, is respectfully urged.

Respectfully submitted

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It is hereby certified that this is being mailed on January 8, 2002.

Francene Sawyer

Means [of tumour therapy] **0107-032**

[Description] Tumor Treating Composition

[The invention in question] **Field of the invention**

The present invention relates to a pharmaceutical [agent on the basis of acombination of anti-oestrogen] **composition of an antiestrogen**, alkylphospholipids and phospholipids, its manufacture and use.

[Fields of application of the invention are medicine and the pharmaceutical industry. In medicamentous tumour] **Background**

In tumor drug therapy, optimal treatment is repeatedly inhibited by the occurrence of resistance against the [pharmacon] **drug** and by toxic side [-]effects. [A part] **Some** of these undesired effects can be [cancelled] **eliminated** or [soothed] **reduced** by encapsulation of the [medicaments] **drugs** in liposomes (D. D. Lasic and D. Papahadjopoulos, Medical Applications of Liposomes, Elsevier, 1998). Liposomal anthracyclins have [reached the stage of extended] **been employed in numerous** clinical [application] **applications**. Specific benefits result if phospholipids with an inherent [anti-tumour]

antitumor effect are used to form the liposomes, e.g. alkyl phospholipids (Arndt et al. Drugs of Today 1998, 34, 83-96).

Alkyl phospholipids are ~~[a]~~ **relatively** new type of ~~[compound,]~~ **compounds,** the ~~[effect]~~ **effects** of which ~~[against tumour]~~ **on tumor** growth is achieved by **their** effects on the cell membrane (Alkylphosphocholines: An update, Drugs of Today, Vol. 34, Suppl. F, 1998). Under certain conditions, alkylphospholipids ~~[result in supra-molecular]~~ **have supramolecular** structures, ~~[inter alia]~~ **such as** liposomes, with more ~~[favourable]~~ **favorable** properties ~~[compared with]~~ **than** the monomeric or micellar ~~[organized compound (DE 41 32 345 A1, DE 44 08 011]~~ **compound (German patents Nos. 4,132,345 A1; and 4,408,011** C1). Further substances ~~[with anti-neoplastic]~~ **having an antineoplastic** effect can **also** be included in these liposomes ~~[with an inherent anti-tumour]~~ **that have an antitumor** effect (Arndt et al., Breast Cancer Res. Treatm. 43 (1997) 237-246, ~~[DE 44 08 011 C1).~~ ~~]~~**German patent No. 4,408,011 C1).**

[Mamma carcinomas, the most frequent tumour in women,] **Breast cancer is the most frequently occurring tumor in women. It** can be influenced in [about 75% of the] **most** cases by endocrine measures**, as can also other cancers such as of the prostate, uterus, brain, and thyroid cancers**. Competitive hormone therapy [by means of Tamoxifen] **with tamoxifen** is of particular importance in this context; in it, the endogenous hormones are [antagonised] **antagonized** at the receptor. Treatment with [Tamoxifen,] **tamoxifen,** which [is low in] **has only a few** side-effects, is however limited by development of resistance against the [pharmacon] **drug**. The causes of [the] **this** resistance [are, inter alia,] **include** alterations of the ligand and its binding to the [oestrogen] **estrogen** receptor (ER), loss or alteration of the ER, alterations of transcription factors or the ER-associated protein or blockage through anti-[oestrogen] **estrogen** binding proteins (Katzenellenbogen et al., Breast Cancer Res. Treat. 44 (1997) 23-38; Osborne, New Engl. J. Med. 339 (1998) 1609-18; [US005904930A).] **US patent No. 5,904,930).**

~~[The objective of the invention is the creation of a medication formulation on the basis of anti-oestrogen, alkylphospholipid and phospholipids.]~~ **Brief description of the invention**

It is an object of the present invention to provide an antineoplastic alkylphospholipid in combination with an estrogen in a lipid vesicle (i.e. a liposome) which is effective in ~~[anti-oestrogen]~~ **antiestrogen** resistant ~~[tumours]~~ **tumors** and which ~~[minimises]~~ **minimizes** or prevents the development of resistance.

The ~~[invention is characterised by the primary claims, the sub-claims being preferred variants]~~ **present invention is a pharmaceutical composition which comprises a combination of an antineoplastic alkyl-phospholipid, a water -or lipid-soluble antiestrogen in a lipid vesicle, and a phospholipid, such as phosphatidylcholine, that has no antineoplastic properties. The composition can optionally also include a cholesterol or other sterol, a lipid with a positive or negative charge, and a polyethylene glycol-modified PEG lipid and/or pharmaceutical carriers and/or excipients.**

[.] **Brief description of the drawing**

The sole figure of this application shows the cytotoxic effect of tamoxifen liposomes on breast cancer cells.

Detailed description

The essential feature of the invention is ~~[the combination of alkylphospholipid with an anti-neoplastic effect and an anti-oestrogen]~~ **a composition which contains an antiineoplastic alkylphospholipid, and an antineoplastic antiestrogen** in a lipid vesicle. A ~~[preferred]~~ **suitable** example **of these ingredients** is octadecyl-(N,N-dimethylpiperidin-4-yl)-phosphate (OPP), ~~[Tamoxifen (Tam) in phosphocholine (PC) vesicles.]~~ **hexadecylphosphocholine, erucylphosphocholine, octadecylphosphoethanolamine, and hexadecylphosphoserine.**

~~[In detail, the agent according to the invention is characterised by the following composition:~~

~~]~~**More particularly, the composition of the present invention contains (a)** an alkylphospholipid **with antineoplastic effect, (b) a water -or lipid-soluble antiestrogen in a lipid vesicle, and (c) an antineoplastically inert phospholipid, and optionally (d) one or more of**~~[(with anti-neoplastic effectivity)~~

~~- a water or lipid-soluble anti-oestrogen with anti-neoplastic effectivity~~

~~- an anti-neoplastically inert phospholipid~~

~~- if need be,]~~ cholesterol or any other suitable sterol, **and**~~[~~

~~- if need be,]~~ a lipid with positive or negative surface charge**, and**~~[~~

~~- if need be,]~~ a polyethylene glycol modified lipid (PEG lipid)**, and further actives as well as a pharmaceutically conventional carrier and/or excipient.**

As used herein, "antineoplastically inert" means a compound that has no antineoplastic properties.

The alkylphospholipids of the present composition suitably has the formula **R-Y-P-X** **(1)**

wherein

R is a C_{12-22}

~~- if need be, further active agents and pharmaceutically customary carrier and ancillary materials.~~

~~Alkylphospholipids with an anti-tumour effect of general structure I are used as phospholipid analogs.~~

~~Structure I: R-Y-P-X~~

~~This formula contains the following meanings:~~

~~R: an~~ alkyl, alkenyl or alkinyl residue ~~[with 12 to 22 C atoms]~~**;**

~~[Y:]~~ **Y is** oxygen, ~~[sulphur]~~ **sulfur** or **a** CH_2 **residue;**

P ~~[:]~~ **is a** phosphate group (PO_2)**; and**

X ~~[:]~~ **is** a choline ~~[or]~~**,** modified choline ~~[rest]~~ **residue** or serine, ethanolamine, glycerine ~~[groups or synthetic modifications of these groups such as the piperidine-4-yl group]~~ **group, or a synthetic modification of the foregoing groups.**

~~[Preferred compounds are]~~ **Suitable examples of X include** hexadecylphosphocholine, octadecylphosphocholine, erucyl- phosphocholine,

octadecyl-[2-(N-methylpiperidinio)ethyl]-phosphate, octadecylphospho-ethanolamine and hexadecylphosphoserine. **A suitable example of a synthetic modification is the piperidine-4-yl group.**

A ~~[The]~~ water or lipid-soluble ~~[anti-oestrogen]~~ **antiestrogen** associated with the phospholipid analogs ~~[is represented by Tamoxifen, Droloxifene, Toremifene, Idoxifene, Raloxifene, Miproxifene-Phospat]~~ **of Formula (I) is suitably tamoxifen, droloxifene, toremifene, idoxifene, raloxifene, miproxifene-phospate** (TAT-59), ICI 1643,384, ICI 182,780 and the main metabolites of ~~[Tamoxifen,]~~ **tamoxifen, namely** 4-hydroxytamoxifen and N-~~[desmethyltamoxifen.]~~**desmethyl-tamoxifen.**

~~[Phospholipids]~~ **Antineoplastically inert phospholipids** without their own ~~[anti-neoplastic]~~ **antineoplastic** effect are **generally** lipids from natural sources or of synthetic origin such as **are** customarily used for liposome production, ~~[e.g.]~~ **for example** phosphatidylcholine.

[Preferably,] **Suitably** polyethylene glycol modified phosphatidylethanolamine in the molecular weight range of 1000 - 6000 Dalton is used as a PEG lipid. [Inter alia, 1,2-Distearoyl] **For example, suitable compounds include** **1,2-distearoyl**-s,n-glycero-3-phosphoethanolamine-N-polyethylenglycol, MG2700; (PEG_{2000}DSPE) and 1,2-[Dipalmitoyl] **dipalmitoyl**-sn-glycero-3-phosphoethanolamine-N-polyethylenglycol, MG5750 (PEG_{5000}DPPE) [are suited. The use of compounds]. **Compounds** which are simultaneously a PEG lipid and an anti-neoplastically effective phospholipid analog [is], **are** also [beneficial, for example] **useful, such as** hexadecylphosphoethanolamine-N-[polyethylenglycol] **polyethyleneglycol**.

According to the invention, **suitably** an anti-neoplastically inert lipid of a natural or synthetic origin is [preferably] used as a base lipid for the membrane formation, such as phosphocholine, serine, ethanolamine, glycerol or other similar lipids, with the ratio of lipid to [anti-oestrogen] **antiestrogen** being **from** 0 [-]**to** 10 : 1 (mass ratio m/m).

[Preferably] **Suitably**, cholesterol or another suitable sterol such as sitosterol is [contained,] **used** with the sterol being in a mol ratio of **from** 0 [:]**to** 1 : 1 to the alkylphospholipid. [

]The liposomal form [preferably comprises] **is suitably a** single-layered or [multi-layered vesicles] **multilayered vesicle** or the liposomes are available as ["] **a** reverse evaporation [vesicles"] **vesicle**.

The effect of the agent [of overcoming] **to overcome** resistance according to the **present** invention can be [proven] **shown** both *in vitro* and *in vivo*. The [means of tumour therapy according to the]**composition of the present** invention is pharmaceutically stable, physiologically outstandingly tolerable**,** and **is** particularly [suitable] **suited** for intravenous application. Undesired metabolism of the [anti-oestrogens] **antiestrogens** is avoided or reduced, **and** improved resorption and distribution of the [pharmacon] **drug** is achieved. [Anti-oestrogens] **Antiestrogens that are** difficult to dissolve in water can [well] be **easily** applied in a liposomal form. The [means] **composition of the present invention** is therefore [outstandingly] **very well** suited for application in [tumour] **tumor** therapy.

The invention is ~~[explained by]~~ **further illustrated through** the following examples~~[:]~~**.**

Example 1:

4.62 mg octadecyl-(1,1-dimethyl-piperidino-4-yl)-phosphate (OPP; 10 µmol), 0.387 mg Z-4-hydroxy-~~[Tamoxifen]~~ **tamoxifen** (HO-Tam, 1 µmol), 1.55 mg cholesterol (4 µmol), and 1.1 mg dicetylphosphate (DCP; 2 µmol) are completely dissolved in 25 ~~[ml]~~ **m**ℓ chloroform/methanol (7/3; v/v) and the solvent **is** then completely evaporated on a rotation evaporator. The finely distributed lipid film ~~[gained is re-suspended]~~ **that is obtained is resuspended** with 1 ~~[ml]~~ **m**ℓ of phosphate-buffered salt solution (PBS, pH 7.4) and intensively moved for at least 3 hours at room temperature on a vibration machine following addition of some glass pearls. The **resulting** suspension of ~~[multi-layered]~~ **multilayered** vesicles (MLV) ~~[obtained]~~ is then repeatedly extruded through polycarbonate filters~~[,]~~ **of a** pore diameter **of** 100 nm, with a LiposoFast basic system ~~[(]~~**(sold by** Avestin, Inc. Ottawa, Canada) until vesicles with an average diameter around 100 nm with a unimodal distribution of sizes and a polydispersity index of less than 0.2 ~~[(]~~**(as determined by** Dynamic Light Scatter Measurement, DLS) are obtained.

The content of OPP, HO-Tam, CH and DCP is checked by ~~[means of]~~ HPTLC. ~~[Above]~~ **Over** 85 % of the original amount is retained. The composition of the liposomes is unchanged compared with the original composition (deviation < 5%). These HO-Tam liposomes are ~~[preferably]~~ **most suitably** used for *in vitro* ~~[examinations]~~ **tests**.

Example 2:

~~[.]~~ 36 mg OPP, 72 mg ~~[Tamoxifen]~~ **tamoxifen** citrate (Tam), 144 mg phosphatidylcholin (PC) and 8.5 mg DCP are completely dissolved in 100 ~~[ml]~~ **m**ℓ chloroform/methanol (7/3; v/v) and the solvent then completely evaporated on a rotation evaporator. The **resulting** finely distributed lipid film ~~[gained re-suspended]~~ **is resuspended** with 12 ~~[ml of]~~ **m**ℓ citric acid/phosphate buffer (pH 6.08), and intensively moved for at least 3 hours at room temperature on a vibration machine following addition of some glass pearls. An MLV suspension is obtained, which is heterogeneous **and** in its size ~~[composition with]~~ **distribution has** vesicle diameters of between 100 and 5000 nm.

These Tam liposomes are ~~[preferably]~~ **most suitably** used for *in vitro* ~~[examinations]~~ **tests** and as initial liposomes for vesicles of a defined size.

Example 3

36 mg OPP, 72 mg ~~[Tamoxifen]~~ **tamoxifen** citrate (Tam), 144 mg phosphatidylcholine (PC) and 8.5 mg DCP and ~~[additionally]~~ 9.7 mg N-(O-methyl-polyethylenglycyl)-1,2-distearyl-s,n-glycero-3-phosphoethanolamine (PEG_{2000}DSPE) are completely dissolved in 100 ~~[ml]~~ **mℓ** chloroform/methanol (7/3; v/v) and the solvent then completely evaporated on a rotation evaporator. The **resulting** finely distributed lipid film ~~[gained is re-suspended]~~ **is resuspended** with 12 ~~[ml]~~ **mℓ** of citric acid/phosphate buffer (pH 6.08) and intensively moved for at least 3 hours at room temperature on a vibration machine following addition of some glass pearls. An MLV suspension is obtained, which is heterogeneous in its size ~~[composition with]~~ **distribution has** vesicle diameters of between 100 and 5000 nm. These Tam liposomes are ~~[preferably]~~ **most suitably** used for *in vitro* ~~[examinations]~~ **tests** and as initial liposomes for vesicles of a defined composition.

Example 4:

Tam MLV's from ~~[example]~~ **Example** 2 are repeatedly extruded through polycarbonate filters, pore diameter 200 nm, with a LiposoFast basic system (Avestin, Inc. Ottawa, Canada) until a unimodal size distribution around 180 nm is achieved with a poly-dispersity index of less than 0.35 (Dynamic Light Scatter Measurement, DLS).

The content of OPP, Tam, CH and DCP is checked by ~~[means of]~~ HPTLC. A liposome suspension containing about 75 % of used Tam and 98 % of OPP is obtained. In addition, the composition of the liposomes is unchanged compared ~~[with]~~ **to** the original composition (deviation < 5%). These Tam liposomes are ~~[preferably]~~ **most suitably** used for *in vivo* ~~[examinations]~~ **tests**.

Example 5:

Peg-Tam MLV's from ~~[example]~~ **Example** 3 are repeatedly extruded through polycarbonate filters, pore diameter 200 nm, with a LiposoFast basic system (Avestin, Inc. Ottawa, Canada) until a unimodal size distribution around

185 nm is achieved with a poly-dispersity index of less than 0.33 (Dynamic Light Scatter Measurement, DLS). [

]The content of OPP, Tam, DCP und Peg_{2000}DSPE is checked ~~[by means of]~~ **with** HPTLC. A liposome suspension containing about 75 % of used Tam and 98 % of OPP is obtained. In addition, the composition of the liposomes is unchanged compared with the original composition (deviation < 5%). The Peg-Tam liposomes are ~~[preferably]~~ **most suitably** used for *in vivo* ~~[examinations]~~ **tests**.

Example 6:

HO-Tam liposomes from ~~[example]~~ **Example** 1 are diluted with **an** RPMI medium with 10% ~~[foetal]~~ **fetal** calves' serum (without added indicator, with ~~[adriamycin/streptomycin) in such a way]~~ **adriamycin/ streptomycin) so** that a concentration of 200 ~~[nmol/ml]~~ **nmol/mℓ** of OPP is reached, then ~~[being]~~ further serially diluted down to 0.78 ~~[nmol/ml]~~ **nmol/mℓ**. The concentration of HO-Tam active agent is then accordingly **from** 20 ~~[nmol/ml]~~ **nmol/mℓ** to 0.08 ~~[nmol/ml.]~~ **nmol/mℓ.**

[The breast] **Breast** cancer cells MCF7, which are sensitive [towards Tamoxifen] **tamoxifen**, and MCF7-R, which are resistant to [anti-oestrogen] **antiestrogen**, are seeded into 96-well plates with a density of $2x10^4$ cells/well and incubated on the following day with HO-Tam liposomes, control liposomes of the composition of the HO-Tam liposomes, but without HO-Tam, HO-Tam[,] dissolved in DMSO and DMSO of the same amount as needed to dissolve the HO-Tam, for three days. [After this, the] **The** supernatants are **then** removed, the cells washed with PBS and then the cell growth inhibition determined with the MTT assay. [For this, the] **The** cells are incubated **for this** with 200 [µl]µℓ MTT solution (4,6-dimethylthiozol-2-yl-2,5-diphenyl-tetrazolium; 0.5 [mg/ml)] **mg/m**ℓ**)** for 4 hours at 37°C, 170 [µl]µℓ of the supernatant **is** carefully removed and the precipitated formasan crystals completely dissolved with a 70% [Isopropyl] **isopropyl** alcohol solution by intensive pipetting and shaking. After this, the 96-well plates are photospectroscopically measured at 540 nm and the growth inhibition calculated in comparison to the growth of untreated cells. A growth inhibition as portrayed in Figure 1 is obtained.

Example 7

~~[~~1 X 10^5 cells/mℓ were incubated with the corresponding liposomes (L), HO-TAM/DMSO and with DMSO for 3 days. The living cells were determined with the MTT assay. The concentration of active agent necessary to inhibit the cell growth by 50% (IC_{50}) is stated.

Tam liposomes according to Example 4 are used for the *in vivo* **treatment** test. As a ~~[tumour]~~ **tumor** model, breast cancer 3366/Tam is transplanted onto female NMRI nude mice and the treatment started when the ~~[tumour]~~ **tumor** is palpable. The animals are given one dose of liposomes with 50 mg/kg Tam (and correspondingly 25 mg/kg OPP) twice a day for 4 weeks. As controls, liposomes containing no Tam are administered, in addition one group being treated with free Tam. The ~~[tumour]~~ **tumor** growth in relation to the control group (physiological salt solution) is determined and portrayed as a percentage T/C ~~[figure in Table 1.]~~, **as shown in Fig. 1 and in Table 1. The example of Fig. 1 shows that 1 x 10^5 cells/ml were incubated with the corresponding liposomes (L), HO-TAM/DMSO and with DMSO for 3 days. The living cells were determined with the MTT assay. The concentration of active agent**

necessary to inhibit the cell growth by 50% (IC_{50}) is represented. The asterisk * means that the result is significantly different from HO-TAM; and a plus sign + means that the r4esult is: significantly different from MCF7(R-).

Table 1~~[~~

~~]~~:

Therapeutic effectivity of ~~[~~Tamoxifen~~]~~ **tamoxifen** liposomes compared with the resistant breast cancer ~~[~~tumour~~]~~ **tumor** 3366/Tam

Group	Substance	Dose, Tam/Lipid	Alteration of body weight	T/C
		mg/kg/injection	% (day 29/51)	%
A	Solvent		3	
B	~~[~~Tamoxifen~~]~~ **tamoxifen**	50/0	-5	91
C	~~[~~Tamoxifen~~]~~ **tamoxifen** liposomes	50/25	-5	63*
D	~~[~~Control~~]~~ **control** liposomes	0/25	-4	88

~~[* Significantly different from Tamoxifen and the solvent control ($p \leq 0.05$)]~~

~~[Patent claims]~~* **Significantly different from Tamoxifen and the solvent control ($p < 0.05$)**

Abstract of the disclosure

A composition containing an antineoplastic alkylphospholipid, and an antineoplastic antiestrogen in a lipid vesicle; more particularly, the composition is a liposome composition which comprises (i) an antineoplastic alkylphospholipid, (ii) an antineoplastic water -or lipid-soluble antiestrogen associated in liposomal form with the **antineoplastic alkylphospholipid, (iii) a nonneoplastic phospholipid, and optionally one or more of (iv) a sterol, (v) a positively or negatively charged lipid, and (vi) a PEG lipid.**